

Amino acid derivatization and analysis in five minutes

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A convenient method permits amino acid separation by capillary gas chromatography in minutes — the suitable analytic forms are prepared in seconds.

Amino acid separation: Gas chromatography

Among present methods of amino acid determination by both gas-liquid chromatography (GC) and reversed-phase liquid chromatography (LC) after a precolumn derivatization, there is no one method which combines rapid sample pretreatment with rapid analysis of the derivatized forms. Because in LC analysis not all the active hydrogen-containing groups need to be treated and condensation of amino group is by far the prevailing approach, the average time for sample pretreatment is shorter than in GC.

Derivatization methods have been reported with time requirements ranging from nearly instantaneous derivative formation with chloroformates with a bulky fluorene moiety [1,2], over one to two minutes requirement for treatment with the popular *o*-phthalaldehyde [3,4], to several minutes for derivatization with, e.g. phenylisothiocyanate [5,6]. With the last mentioned reagent the most rapid analysis of a complete mixture of protein amino acids was achieved, in 12 min, which is the shortest time for determination of amino acids by LC. However, 60 min were necessary for physiological samples of amino acids [5,7] using the same method and the one hour analysis time can be considered as an average for LC performed amino acid analyses. Unlike GC analysis, use of capillary columns in LC lengthens the total time of a chromatographic run [8,9]. When considering GC in connection with amino acid analysis rather the opposite is to be expected; rapid analysis, but slow derivatization [10]. The widely used esterification-acylation procedures require on average one hour

of sample handling, the recently introduced silylation procedure even more [11]. Shorter time of treatment — about 20 min — were achieved with isobutyl chloroformate [12] and dichlorotetrafluoroacetone [13]. Moreover, the latter procedure enabled us to analyse 20 protein amino acids in less than 10 min, which was the shortest time of amino acid analysis of all. With short and narrow bore capillaries time below 10 min should be reachable without sacrificing the quality of resolution, as was demonstrated just recently [14]. This flexibility and speed of analysis are strong points of GC in comparison with LC.

When summarizing criteria aiming to the establishment of a hypothetical, ideal procedure of amino acid determination, the following are required: (a) simple sample handling with possibly one reagent only, (b) ability to derivatize in aqueous medium, (c) an instantaneous or very rapid reaction course proceeding at room temperature, (d) few-minute analysis with a good resolution and (e) low reagent and instrumental costs. Reviewing the nearly 40 years spent on amino acid methodology it would be rather unrealistic to expect fulfilment of all these requirements in one single procedure.

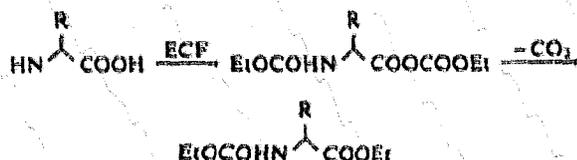
But miracles happen. With the procedure reported here we are close to that ideal. The derivatization may no longer be the Achilles heel of GC. The chemical pretreatment of the sample determines the rapidity of analysis. This method takes about one minute and requires only five microliters of an inexpensive reagent. And the chemical conversion tolerates water, even dilute hydrochloric acid.

The procedure is based on the treatment of aqueous amino acid solutions with ethyl chloroformate (ECF). An approach using isobutyl chloroformate to the same purpose has already been reported [12] but, as mentioned in another report [15], an additional reaction step was necessary for the treatment of the carboxylic

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Amino acid abbreviations used: A, alanine; G, glycine; V, valine; L, leucine; I, isoleucine; T, threonine; S, serine; E, glutamic acid; P, proline; N, asparagine; D, aspartic acid; M, methionine; OH-P, hydroxyproline; F, phenylalanine; C, cysteine; Q, glutamine; O, ornithine; K, lysine; H, histidine; Y, tyrosine; W, tryptophan; C-C, cystine

group. Under optimal composition of the reaction medium, however, even the carboxylic group can be esterified, via decarboxylation of the intermediate mixed anhydride form:



Next, the amino acids are converted into and analyzed as *N*-(*O,S*)-ethoxycarbonyl ethyl esters.

The simple chemical treatment is as follows: amino acid residue (less than about 100 μg in total) is treated with 100 μl of water/ethanol/pyridine, 60:32:8 solution, or, alternatively 60 μl of weakly acidic (20-25 mmol/l HCl) aqueous amino acid solution are mixed with 40 μl of ethanol/pyridine (4:1) solution, and then 5 μl of ECF are added. The tube is shaken gently for about 5 s during which foaming due to gas evolution (decomposition of the reagent to alcohol and carbon dioxide) usually appears. 100 μl of chloroform (containing 1% of ECF) are added and extraction of the derivatives proceeds preferably by striking the vial against a pad for about 5 s. An aliquot of the chloroform layer (not necessarily the lower one) is injected in GC.

An HP 5890 gas chromatography with flame ionization detector and split injection mode (1:20) was used for analysis of equimolar mixtures of amino acids as presented in Fig. 1. The moderately polar OV-1701 polysiloxane phase proved to be the best phase for an effective separation of the derivatized compounds in less than 5 min. Optimal results were obtained with 10 m \times 0.25 mm I.D. fused silica column CP-Sil 19 CB (Chrompack, The Netherlands) with an immobilized phase layer of 0.2 micron. Equivalent results with regard to separation and time were obtained with

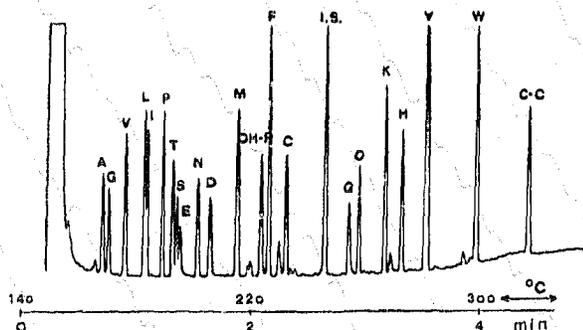


Fig. 1. GC-FID analysis of an equimolar mixture of the specified amino acids after treatment with ECF on the OV-1701 capillary. Temperature raise 40°C/min, hydrogen head pressure 50 kPa. The injected amount represents 25 pmol out of 25 nmol of each amino acid derivatized.

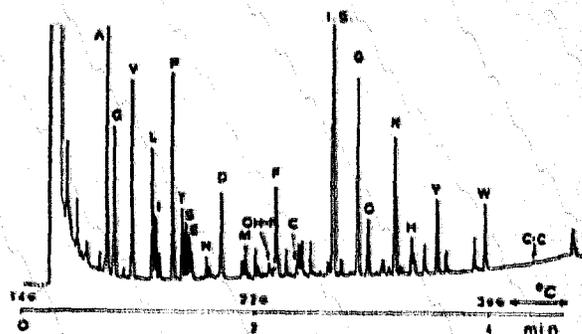


Fig. 2. Chromatogram of amino acids isolated from 50 μl of human serum as described and derivatized with ECF. Equal column and chromatographic conditions as in Fig. 1 were used. As the internal standard, *p*-chlorophenylalanine in the amount of 10 nmol was added to the serum before isolation.

hydrogen and helium as carrier gasses, provided the pressure was doubled (100 kPa) with helium.

The conditions for derivative formation and column separation are optimal. By changing, e.g. the composition of the medium it is possible to promote higher derivatization yields for some amino acids but at the cost of others. The antagonistic behaviour involves basic vs hydroxy aliphatic amino acids. Double derivative formation was observed with glutamic acid and ornithine (their accompanying peaks are eluted behind those of M and F), their ratio can be partially influenced by acidity of the reaction medium. This is the reason for recommended use of diluted hydrochloric acid instead of water alone. Addition of ECF into chloroform improves the yields of 3 amino acids (N, D and C) substantially. Within-day reproducibility of derivative formation, with respect to the values of molar ratios (*p*-chlorophenylalanine used as internal standard) was good, with variation coefficients below 5%, with exception of E, Q and H (6-8%). From the chromatographic point of view improvement of separation of I-L and S-E pairs is not possible simultaneously as better separation of one of those pairs is accompanied by worsening in separation of the other. Thus, the chosen conditions of temperature programming are not only because of the speed but because of the optimal separation. For details on these items see parallel report [16].

The presented method has a weak point as well; the inability to elute arginine from the column or from any of the columns tested. The reason is known: the guanidine group is very prone to absorption in the column, because the action of chloroformates leaves the imino group untouched. This was already observed earlier [12]. For determination of arginine it would be, therefore, necessary to develop an additional reaction step.

Applicability of the method and sufficient separation power of the column for analysis of amino acids from,

e.g. human serum, is documented in Fig. 2. The compounds of interest were isolated from the biological source simply without deproteinization, by percolating the serum diluted with 25% acetic acid in ratio 1:10 through a cation exchanger (Dowex 50W×2) in a micropipette tip, essentially according to our earlier report [17]. Including evaporation of the aqueous ammonia (250 µl of eluate volume) the whole isolation procedure can be carried out in 15 min.

The presented procedure of amino acid determination is unique in rapidity of sample preparation and total time of analysis. It has no equivalent among all known procedures regardless the technique used. It may pose a challenge to all who are interested in simple and rapid amino acid determination.

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